1988

Callus Formation and Differentiation in Embryos of the Coconut Palm (Cocos nucifera L.)

Jyoti Mangesh Desai
Eastern Illinois University

THESIS REPRODUCTION CERTIFICATE

TO: Graduate Degree Candidates who have written formal theses.

SUBJECT: Permission to reproduce theses.

The University Library is
institutions asking permis
in their library holdings.
feel that professional cou
from the author before we

Please sign one of the fol

Booth Library of Eastern
my thesis to a reputable
it for inclusion in that ins

August 11, 1988

Date

I respectfully request Bo
allow my thesis be repro

Date

LIBRARY - Name & Address

1406

Bound By AMERICAN BINDERY, Topeka, Kansas.

TRIM SIZE	1 2 3 4 5 6 7 8 9 10
Buckram Color # 538	Your Spine will be lettered **EXACTLY** as it appears on your Binding Slip.
Stamp in White ☒	**Callus Formation and Differentiation in Embryos of the Coconut Palm (Cocos Nucifera L.)**
Stamp in Black ☐	
Stamp in Gold ☐	
NEW: ☐	
Bound Before ☐	
Rub Enclosed ☐	
Sample ☐	
ADS:	**Desai**
Leave In ☐	
Take Out ☐	
COVERS:	
Remove ☐	**LB 1861 .C57x D441 cop.2**
Bind in All ☐	
Bind in Front ☐	
_____ ☐	
INDEXES:	
Front ☐ Stub For ☐	
Back ☐ No Index ☐	
SPECIAL INSTRUCTIONS:	

BINDERY COPY

m

CALLUS FORMATION AND DIFFERENTIATION IN EMBRYOS OF THE COCONUT PALM (*COCOS NUCIFERA* L.)

(TITLE)

BY

JYOTI MANGESH DESAI

M.S., UNIVERSITY OF BOMBAY, 1984

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF SCIENCE

IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
CHARLESTON, ILLINOIS

1988
YEAR

I HEREBY RECOMMEND THIS THESIS BE ACCEPTED AS FULFILLING
THIS PART OF THE GRADUATE DEGREE CITED ABOVE

Aug 11 1988
DATE

August 16, 1988
DATE

ABSTRACT

Desai, Jyoti M. M.S., Eastern Illinois University. August 1988. Callus formation and differentiation in embryos of Cocos nucifera L. Major Professor: Dr. William A. Weiler.

Embryos of Cocos nucifera L. were allowed to develop on various agar based, plant tissue culture media. Environmental conditions, temperature, light intensity levels and photoperiod were regulated throughout the period of the experiment. The anatomy of commercially available embryos (excised from the "seed") and tissues that developed in culture (calli, roots and leaves) was determined. Prepared slides and tissues obtained in culture were photographed and printed. Callus, root and shoot formation were successful. However, "embryoids" which would have eventually led to free living plantlets were not obtained during this study. Successful regeneration of plantlets in culture would require improved techniques.

ACKNOWLEDGEMENTS

The author wishes to express her sincere gratitude to Dr. William A. Weiler for his constant advice, encouragement and guidance, without which this research would not have been possible. I would like to thank Dr. Steven A. Becker for his suggestions on the plant anatomy aspects of this paper. I wish to thank Dr. Terry M. Weidner and Dr. John M. Speer for their helpful suggestions in the completion of this paper. I wish to thank Mr. Lawrence E. Crofutt for his photography. A special thanks goes to Dr. Andrew S. Methven for his technical assistance in the photography department, and to Kevin Lyman for his continuous support. Last but not the least, to my family who always said that one can do anything in this world if one put his mind to it.

ABSTRACT

Desai, Jyoti M. M.S., Eastern Illinois University. August 1988. Callus formation and differentiation in embryos of Cocos nucifera L. Major Professor: Dr. William A. Weiler.

Embryos of Cocos nucifera L. were allowed to develop on various agar based, plant tissue culture media. Environmental conditions, temperature, light intensity levels and photoperiod were regulated throughout the period of the experiment. The anatomy of commercially available embryos (excised from the "seed") and tissues that developed in culture (calli, roots and leaves) was determined. Prepared slides and tissues obtained in culture were photographed and printed. Callus, root and shoot formation were successful. However, "embryoids" which would have eventually led to free living plantlets were not obtained during this study. Successful regeneration of plantlets in culture would require improved techniques.

TABLE OF CONTENTS

INTRODUCTION.....1

LITERATURE REVIEW.....3

MATREIALS AND METHODS.....12

RESULTS AND DISCUSSION.....16

CONCLUSION.....39

LITERATURE CITED.....40

LIST OF PLATES AND TABLES

Plate | Page

1. Line drawing of a longitudinal section of the embryo of Cocos nucifera showing the positions of the haustorium (H) and apical meristem (AM) 18

2. Line drawing of a cross section of the embryo of Cocos nucifera showing positions of the haustorium (H) and apical meristem (AM) 18

3. Photograph of a longitudinal section of the embryo of Cocos nucifera, showing nodular indentations (IN) 20

4. Photograph of a longitudinal section of the embryo of Cocos nucifera, showing procambial strands (PS) 20

5. Photograph of a longitudinal section of the embryo of Cocos nucifera showing the apical meristem (AM) and leaf primordia (LP) 22

6. Photograph of a cross section of the embryo of Cocos nucifera showing apical meristem (AM) and leaf primordia (LP) 22

7. Photograph of a compact callus (C) accompanied by browning (BR) (Bar = 1cm) 25

8. Photograph of callus in culture showing separated globular structures (GS) and fingerlike projections (FP) 25

9. Photograph of callus in culture showing fingerlike projections (FP), rootlike outgrowths (RO), and tissue browning (BR) (Bar = 1cm) 27

10. Photograph of a portion of a cross section of callus showing a mass of cells (C) and peripheral stained cells (SC) 27

11. Photograph of callus showing differentiation into rootlike outgrowths (RO) (Bar = 1cm) 29

12. Photograph of callus in culture producing a root (R) (Bar = 1cm) 29

13. Photograph of a longitudinal section of a root produced in culture showing procambial strands (PS) 31

14. Photograph of a longitudinal section of a root produced in culture showing apical meristem (AM) and root cap (RC) 31

Plate Page

15. Photograph of callus in culture differentiated into leaves (L) (Bar = 1cm).....34

16. Photograph of a cross section of a leaf produced in culture showing epidermis (E), expansion cells (EC), tannin cells (TC), and vascular bundle (VB) 34

17. Photograph of a cross section of a leaf produced in culture , focusing on expansion cells (EC) 34

18. Photograph of embryoid-like structures produced in culture (EM) (Bar = 1cm) 36

19. Photograph of an embryoid-like structure produced in culture (EM) (Bar = 1 cm) 36

20. Photograph of a longitudinal section of the embryoid-like structure showing tracheary elements (T) in cross section 38

21. Photograph of a longitudinal section of embryoid-like structure showing origins of tracheary elements of primary root (T) 38

Table

1. Murashige and Skoog (MS) mineral formulation 13

2. Palm Culture Media 13

INTRODUCTION

Being a very important economic perennial in the tropics, the coconut palm (Cocos nucifera L.) deserves special attention. Every part of the palm is of considerable importance to mankind. It is a major source of oil, food, drink, medicine, fibre, fuel and countless other products. The oil is used in soapmaking, cooking and lubrication. The leaves serve to thatch roofs and make useful mats and baskets. The trunks are used for building purposes, the roots are used as a substitute for toothbrushes, and the endosperm is used in cooking. Coconut milk is a common beverage. The spathe yields "toddy", a source of palm sugar and vinegar. The shells serve as various utensils, and coir, the "husk" of the fruit, is used for insulation and for making ropes, brushes and brooms.

In recent times, emphasis is being placed on increasing the yield of the palm. In spite of its extensive fruit yield, production of superior strains is limited due to various reasons. First, the palm requires a long time for germination and attainment of sexual maturity. Second, the palm is cross fertilized. Third, it is highly vulnerable to diseases and insect pests. Finally, most palms are heterozygous.

Clonal propagation of the palm as a means of vegetative propagation is a recent attempt to increase crop yield. Since the palm has a single shoot apex, vegetative propagation in the form of cutting or grafting is impractical. Attention is currently being focused on using means of (asexual) vegetative propagation and producing a large number of genetically identical plants. This research project is primarily concerned with establishing the conditions necessary for

formation and growth of coconut callus tissue in the laboratory. Callus tissue is undifferentiated; it can be stimulated to differentiate into plantlets by regulating the cultural conditions (physical, nutritional, hormonal) of the growth medium. Coconut palm embryos were chosen for this study because they are relatively undifferentiated, quite large and easy to isolate aseptically, and readily available.

LITERATURE REVIEW

LIFE HISTORY

The coconut palm (Cocos nucifera L.) has a pantropical distribution although it's origin is a controversial topic. The most widely accepted theory of the origin of this palm is that it is from Asia or Polynesia (Beccari, 1917; Corner, 1966; Purseglove, 1972). A theory on the American origin (Cook, 1910) is not supported by the discovery in South Africa of Jubaeopsis caffra Beccari, a close relative of Cocos (Beccari, 1917). The coconut palm was probably transported via ocean currents and occasionally established itself on shores without the aid of man, which would account for it's worldwide distribution (Beccari, 1917).

A cocoid palm, Cocos nucifera L. belongs to the subfamily Cocoideae among the Palmae. The Cocoideae originally included about 60 species. Beccari in 1884 made the genus monotypic by separating it from closely related genera, including Jubaeopsis, Syagrus, Butia, Arecastrum and Jubaea (Corner, 1966).

Ochse et al. (1961) and Purseglove (1972) described the morphology of the palm at length. The mature palm is polymorphous, unarmed, unbranched and extends to a height of 60 to 100 feet. It has a life span of about a century. The roots are adventitious and arise from a swollen basal portion of the stem (referred to as the "bole"). They are approximately 8-10 mm in width and 6 m long. Root hairs are absent. Thin walled epidermal cells present near the root cap are responsible for absorption. Underlying these cells is the hypoderm.

The hypoderm is penetrated by modified rootlets or pneumathodes that arise from the pericycle. The pneumathodes channel oxygen into the internal tissues. Air spaces within the non-fibrous cortical region are well developed. The outer cortex is narrow and has numerous mucilage canals. The inner cortex is sclerotic. The stele is medullated and the phloem cells are large and extend radially (Tomlinson, 1961).

Vascular cambium is absent. A peripheral parenchymatous cambial zone is responsible for bringing about an increase in the width of the columnar trunk. The trunk is composed of a series of alternating nodes that bear leaves. Persistent leaf scars are conspicuous. The internodes are shorter than the nodes. The nodes attain their maximum width with increasing maturity. Surface layers of old stems are sclerotic. The stem cortex is narrow and fibrous and contains few small vascular bundles, while the central cylinder has numerous vascular bundles. The vascular bundles in the stem contain two metaxylem vessels. The peripheral vascular bundles are more compactly arranged than those in the center and the phloem sheaths become sclerotic at maturity (Tomlinson, 1961).

The large, pinnately-compound, petioled leaves are spirally arranged in a terminal crown. The numerous, sessile leaflets are borne on a woody midrib (Ochse et al., 1961). Unique among the palms, in Cocos numerous hairs are present on the abaxial intercostal regions. The base of the hairs is sunken, surrounded by 3-4 swollen epidermal cells that have cutinized walls (Tomlinson, 1961). The epidermis itself is cuticularized with sunken stomates that are restricted to the abaxial intercostal regions. A hypodermis is distinct with indistinct

peripheral chlorenchyma. Stegmata (silica cells) and starch granules are abundant throughout the mesophyll (Tomlinson, 1961).

The palm is monoecious and the spadix is borne in the leaf axil. The male flowers are borne singly and are sessile. The perianth segments are in two whorls of three each, the outer tepals being larger than the inner. Stamens are six in two whorls and the pistil is rudimentary and bears nectaries at its apex. The female flowers are globose. The tepals are of uniform length, six in two whorls. The ovary is trilocular and bears a trifid stigma with nectaries at its base. The fruit is a fibrous drupe, the seed being enclosed within a stony endocarp (Purseglove, 1972).

The coconut palm is cross-pollinated. Pollination is primarily entomophilous, although the dry pollen can be dispersed by wind. The male flowers begin to open first in acropetal succession, and the last flower is shed after about 16-22 days. Around this time, the female flowers begin to open. Each female flower is receptive for 24 hours after the pollen has been shed. The time interval between the opening of the stigma of the first and the last flower is about 5-7 days (Purseglove, 1972).

The fruit ripens about a year after fertilization. Two of the three carpels degenerate within the first 6 months. The embryo sac of the functional ovule enlarges and becomes filled with sap or coconut water. Within the next 6-10 months, the endocarp, embryo and the originally jelly-like endosperm reach maturity (Purseglove, 1972). Growth of the embryo and seedling is continuous as the seed does not undergo a dormancy period.

Palm embryos, in general, are similar except for size. The

partially undifferentiated embryos are white or cream colored. The embryo of the date palm (Phoenix dactylifera) is approximately 2 mm in length (Tisserat and DeMason, 1980), while that of the coconut palm averages between 5-8 mm (Gupta et al., 1984). Studies on the date palm reveal the organization of the embryo into the shoot apex or epicotyl, the cotyledon, and the root pole or hypocotyl, the latter two being separated from the shoot apex by an internal cavity that houses the apical meristem (Tisserat and DeMason, 1980). The thin-walled cells of the shoot tip region are densely cytoplasmic and are smaller than those of the proteinaceous cotyledonary cells. Procambial strands extend from the root pole region to the haustorial end of the cotyledon (Tisserat and DeMason, 1980).

Germination begins with a pronounced lengethening of the cotyledon. One end of the elongated cotyledon carries the embryo through the fertile eye. This is followed by the emergence of the plumule and radicle, which forms the first root. The tip of the growing shoot is protected by scale leaves until it emerges from the exocarp and produces the first simple leaves. The radicle subsequently degenerates and adventitious roots develop from the nodes (Purseglove, 1972). The cotyledonary portion still within the seed forms the haustorium. It grows and completely fills the central cavity. The haustorium is made up of thin-walled cells separated by large intercellular air spaces. Nutrients absorbed from the liquid and solid endosperm are carried to the developing seedling via vascular strands present within the haustorium. The roots also absorb nutrients, essentially potassium, from the mesocarp (Purseglove, 1972).

The palm grows well in saline, well-drained, well-aerated soils

with a pH ranging between 5.0 and 8.0. It requires a constant supply of moving fresh water, high humidity and plenty of sunlight, with an average desirable temperature ranging between 27-32^{o}C (Purseglove, 1972). These conditions need to be met when the drupes are planted in nurseries. The "seednuts" are partly submerged horizontally in the soil; with the fertile eye above the soil surface. They are transplanted after 6 months, either directly into their permanent habitat or in a nursery for another 3-4 years (Ochse et al., 1961). Germination, vigor and growth rate are important factors that cannot be ignored during seedling selection. Seedlings that fail to germinate early are discarded. The seedlings should have straight stems that bear 3 wide, healthy leaves. The seeds should be lifted carefully from the soil during transplanting. Weeds should be controlled and the seeds should be free from pathogens. Since the palm has a long lifespan, seedling selection plays an important role in overall yield and production (Purseglove, 1972).

TISSUE CULTURE

Asexual methods of reproduction such as cutting and grafting are conventional methods attempted to produce "clones" of plants that have a genetic makeup similar to that of the parent. These methods usually produce a single clone at a time, require considerable space and labor, are relatively expensive and are susceptible to deleterious environmental factors, particularly pathogens (Kyte, 1987). Micropropagation of plants under controlled conditions in the laboratory results in the production of a large number of clones within a relatively short time. Such methods involve less expense and labor than conventional methods (Kyte, 1987).

Most economically important plants can now be induced to grow artificially on nutrient media. Any part of a parent plant can be used as an explant to start the culture. Roots, stems, leaf tissues, apical meristems, anthers and embryos serve as good explant materials. Plant embryos are particularly interesting because they form embryoids more easily on proper nutrient media than mature plant tissues (Torrey 1973). Among the monocots, extensive work has been done on orchids and grasses.

Tissue culture of palms, especially that of the coconut, date and oil palms, is a valuable tool for producing improved yields and resistant cultivars. Breeding experiments are made difficult due to the heterozygous nature and long life spans of most palms (Nambiar and Swaminathan, 1960). Seed propagated coconut palms vary widely in overall productivity, vigor, and disease resistance. These traits are often expressed when the palms are ten years old. As a result, seed

selection and breeding is not always foolproof (Branton and Blake, 1983). Hence, the importance of tissue culture as a view to vegetative propagation cannot be overemphasized.

Since the 1960's, research concentrating on the oil, date and coconut palms has made considerable progress. Corley et al.(1977) and Tisserat (1979) regenerated free-living oil palm and date palm plantlets from asexual embryos. On the other hand, limited success has been achieved with experiments on the coconut palm.

The main criteria to be considered for tissue culture experiments are explant material, mineral composition of the medium and prevention of browning of explants (Tisserat, 1984). The plant parts used as explant materials are roots, petioles, leaf tissues, inflorescence rachillae, shoot tips, and embryos. Embryogenic callus initiation is more promising when meristematic tissues are used as explants (Tisserat, 1979). Zygotic embryos from most palms are very efficient sources of embryogenic calli (Tisserat, 1984). Callus derived from stem and inflorescence of coconut (Eeuwens, 1976) and from stem and leaf tissue of date (Eeuwens, 1978) is not embryogenic, and this callus often produces roots only (Tisserat, 1979).

Callus develops from undifferentiated meristematic cells along procambial strands in coconut embryo explants (Guzman et al., 1978) and at root tips of oil palms (Martin et al., 1972) (cited by Paranjothy, 1984). To date, there is no specific nutrient medium for palm tissue cultures. After investigating various nutrient media, Eeuwens (1976) concluded that the Murashige and Skoog (MS) and Y-3 basal mineral formulations are most suitable for callus initiation. These two mineral compositions are rich in nitrogen. The addition of iodine to

these media proved to be beneficial for callus induction in the coconut palm (Eeuwens, 1976). In addition, auxin is a basic requirement for the production of callus *in vitro* (Tisserat, 1979). Vitamins such as thiamine-HCl and i-inositol added to the nutrient medium are helpful (Tisserat, 1979). According to Apavatjrut and Blake (1977), addition of amino acids is not required for callus initiation (Tisserat, 1984). The addition of 5-20% coconut water (CW) enhanced germination of coconut embryos (Cutter and Wilson, 1954). Special requirements for coconut and oil palm embryo germination on a modified MS medium include indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), benzyl adenine (BA), CW, and 2-isopentenyl adenine (2ip) (Fisher and Tsai, 1978).

Embryogenesis from callus tissue takes place in the absence of auxin (Tisserat, 1979). In addition, Rabechault and Martin in 1976 stated that cytokinins were necessary for embryogenesis (Paranjothy, 1984). Increased concentrations of sugar and the addition of naphthalene acetic acid (NAA) to the basal medium induces adventitious rooting (Eeuwens, 1978).

Nutrient requirements for development of plantlets have been investigated. According to Martin and Rebachault (1978), oil palm embryos produced roots and shoots respectively when NAA and sucrose were added to the medium (Paranjothy, 1984). Plantlets were also obtained from embryos cultured in basal media devoid of hormones (Reynolds and Murashige, 1979). Recent reports on the coconut palm include plantlet regeneration from root callus (Branton and Blake, 1983) and plantlet regeneration from embryos cultured on Y-3 liquid medium supplemented with NAA, BA, and CW (Gupta *et al.*, 1984). Not all embryos produced plantlets. Embryos 6-7 mm long and 4 mm wide were

more successful in developing into plantlets (Gupta et al., 1984).

Browning of explants is minimized by addition of activated charcoal to the basal medium. Browning is a result of tissue wounding and the release of phenolic compounds. Apparently, cytokinin and agar in the medium enhance browning (Martin and Rabachault, 1978; cited in Paranjothy, 1984).

Contamination in cultures is a frequent problem which can be minimized by surface sterilization. Various sterilants have been used, such as chloramine-T, peracetic acid, 8-hydroxyquinone, mercuric chloride, alcohol, and sodium hypochlorite. Surface sterilization with 2% sodium hypochlorite appears to be most effective (Tisserat, 1984). Internal contamination may appear after several weeks in culture (Cutter and Wilson, 1954) and is difficult to control, although treatment with antibiotics has been suggested (Fisher and Tsai, 1978).

In order to obtain maximum yield, a knowledge of the growth patterns and biochemistry of a crop is essential. In vitro clonal propagation allows for the analysis of the growth patterns and biochemical nature of crops, including the palms. Current research should be concerned with improving culture techniques, mass cloning, and determining cell division patterns in culture (Tisserat, 1984). Tissue culture can also be used as a tool for clonal propagation and storage of germplasm of a wide variety of economically important crop plants. Palm callus can be preserved at low temperatures using refrigerants, and later revived to produce plantlets (Tisserat et al., 1981; cited in Paranjothy, 1984).

MATERIALS AND METHODS

EXPLANT MATERIAL

Coconut "seeds" were obtained commercially from local grocers. The embryo of the coconut is located at the end of the endocarp showing three dark "eyes". A hole was drilled at the end away from the one where the embryo is situated and the liquid endosperm was allowed to drain. This was done in order that seeds could be easily cracked without splattering. The coconuts were cracked open with a hammer. One of the three indentations was darker and softer on the outside and showed an indentation in the endosperm from the inside. The embryos appear to be embedded in the endosperm at this position. They were thus located, excised, and respectively passed through two alternate changes of sterile distilled water and 2% sodium-hypochlorite solution, followed by three changes of sterile distilled water. The time interval between successive changes was 1 minute. Some of the embryos were cut transversely into five approximately equal pieces. Others were cut into longitudinal halves. Sections were placed on callus-induction medium and incubated at 20^{o}C in the dark in a Precision Scientific Incubator.

MEDIA COMPOSITION

Minerals and growth regulators used for this research are listed in Table 2 (from Tisserat, 1984). All materials required were obtained through Carolina Biological Supply Co. The Murashige and Skoog (MS) mineral formulation (Table 1) was used as the basal medium. A vial of MS salts (enough to prepare 1 liter) was dissolved in 800 ml of reagent grade water using a magnetic stirrer. Sucrose was then

Table 1: Murashige and Skoog (MS) Mineral Formulation

Macronutrients	mg/liter	Micronutrients	mg/liter
$MgSO_4 \cdot 7H_2O$	370	$FeSO_4 \cdot 7H_2O$	27.8
$CaCl_2 \cdot 2H_2O$	440	$MnSO_4 \cdot 4H_2O$	22.3
KNO_3	1990	KI	0.83
NH_4NO_3	1650	$CoCl_2 \cdot 6H_2O$	0.025
KH_2PO_4	170	$ZnSO_4 \cdot 7H_2O$	8.6
		$CuSO_4 \cdot 5H_2O$	0.025
		H_3BO_3	6.2
		$Na_2MoO_4 \cdot 2H_2O$	0.25

Table 2: Palm Culture Media

COMPONENTS	MEDIA TYPES		
	Callus Production	Shoot Tip	Adventitious Rooting
INORGANIC SALTS			
MS Formulation	+	+	+
CARBOHYDRATE SOURCE			
Sucrose (M)	0.087	0.087	0.087
VITAMIN SOURCES			
Meso-Inositol dihydrate	0.46	0.46	0.46
Thiamine.HCl	1.2	1.2	1.2
PHYTOHORMONES			
2,4-D (uM)	440	44.0	-
2iP (uM)	14.5	-	-
NAA (uM)	-	-	0.54
COMPLEX ADDENDA			
Phytagar (%)	0.8	0.8	0.8
Charcoal, activated, neutralized (Sigma) (%)	0.3	0.3	-

added and dissolved. Vitamins (i-inositol and thiamine-HCl) and phytohormones (2,4-dichloro-phenoxy acetic acid, 2 isopentenyl adenine and/or naphthalene acetic acid) were added as needed (see Table 2). The pH was adjusted to 5.7 with 1N sodium hydroxide and the final volume was brought to one liter. Agar was added and heated to dissolve. Care was taken to avoid boiling. Activated charcoal was added and mixed. The medium was dispensed into 25x200 mm tubes, standard petri plates or jars. The containers were autoclaved for fifteen minutes at 15 psi and cooled to 50°C. Slants were allowed to solidify in slanted position.

CULTURE

Extracted and sterilized embryos were allowed to grow on callus-induction medium for approximately 8 to 10 weeks. During this period, the cultures were transferred to fresh callus medium approximately every fifteen days in order to ensure a constant supply of growth hormones.

Callus was subsequently subcultured on shoot induction medium in glass jars kept at 80-90F in a Biotronette Mark III Environmental chamber. The tissues were maintained at a light intensity of 3281.80 lux with a 12hr photoperiod. The relative humidity was supplemented by placing water at the bottom of the chamber. Callus differentiated into root-like structures and outgrowths in about 4 to 6 weeks. Some calli also showed evidence of becoming photosynthetic.

After about 10-12 weeks, root-like outgrowths formed on shoot medium were subcultured on adventitious rooting medium under the same environmental conditions and allowed to produce adventitious roots.

HISTOLOGY

Whole embryos, calli, roots and leaves were fixed in Navaschin's fixative for a period of three days up to eight weeks. The tissues were dehydrated in a tertiary-butyl alcohol series prior to infiltration and embedding in TissuePrep (M.P.60^{o}C, Fisher Scientific Company). Embedded tissues were sectioned at a thickness of 10-12 micrometers in transverse and/or longitudinal planes using a rotary microtome. Sections were serially affixed to glass microslides using Haupt's adhesive followed by staining with both standard safranin and fast-green stains. Permanent slides were prepared by sealing the coverslips with Permount and allowing the slides to dry on a warming plate for 4 to 6 days. Sections were photographed using a Nikon S-KeII microscope, Microflex M-35S photomicrographic unit, and Kodak Panatomic-X film (ASA 32).

Prior to developing and printing the film, all required stock solutions were prepared as follows:

1. Kodak HC-110 developer for black and white film (Dilution B stock solution): 1 part Kodak HC-110 to 31 parts water.
2. Acid stop bath: 3/4 oz. acetic acid (28%) in 16 oz. water.
3. Kodak fixer: full strength.
4. Kodak hypo-clearing agent: stock solution made from packet of powder dissolved in 1 gallon water.

Prints were prepared using Kodak Polycontrast II RC paper and Vivitar enlarger, model E-34.

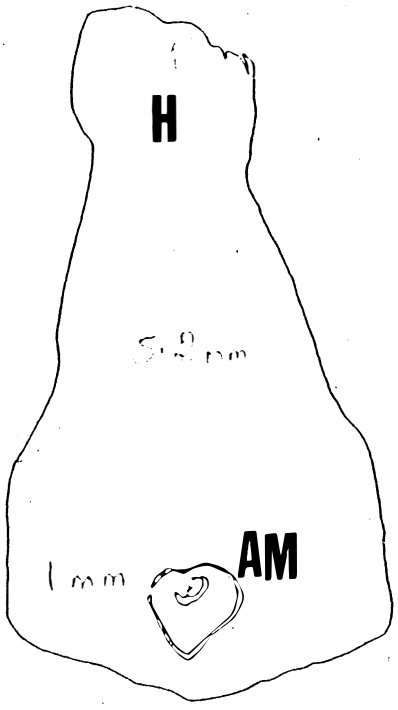
RESULTS AND DISCUSSION

HISTOLOGY OF THE EMBRYO

The excised embryos are approximately 10 mm in length, white in color and situated under the fertile "eye" of the seed. The haustorial end (the distal portion of the cotyledon) is embedded within the endosperm, obtaining nourishment from it. At this stage, the embryo is fully differentiated into the shoot apex (epicotyl), cotyledon, and root pole or hypocotyl (Plates 1 and 2). This is analogous to the structure of the embryo of Phoenix dactylifera (Tisserat and DeMason, 1980). A closeup of the outline of a longitudinal section of the embryo reveals nodular indentations in the haustorial region (Plate 3). These possibly provide for the expanding haustorium which ultimately fills the cavity within the endosperm. The indentations could also provide increased surface area for food absorption. Embryo vascularization is represented by procambial strands which extend from the root pole region to the haustorial end of the cotyledon (Plate 4). The apical shoot meristem is enclosed within a cavity approximately 0.7 mm from the proximal end and 5.2 mm from the haustorial tip (Plate 1). The apical shoot meristem and the leaf primordia are observed about 1 mm from a lateral position in a longitudinal section (Plates 5 and 6). The shoot meristem appears to be closer to the root pole. The cells at the shoot tip region are smaller than those of the rest of the embryo, averaging 10 micrometers in diameter. They are densely cytoplasmic, possibly rich in lipid and protein, thin-walled, and compactly arranged. The cotyledonary cells are larger, average 25 micrometers in diameter, are thick-walled, highly vacuolated and have less cytoplasm.

Plate 1: Line drawing of a longitudinal section of the embryo of Cocos nucifera showing the positions of the haustorium (H) and apical meristem (AM).

Plate 2: Line drawing of a cross section of the embryo of Cocos nucifera showing positions of the haustorium (H) and apical meristem (AM).


H
AM

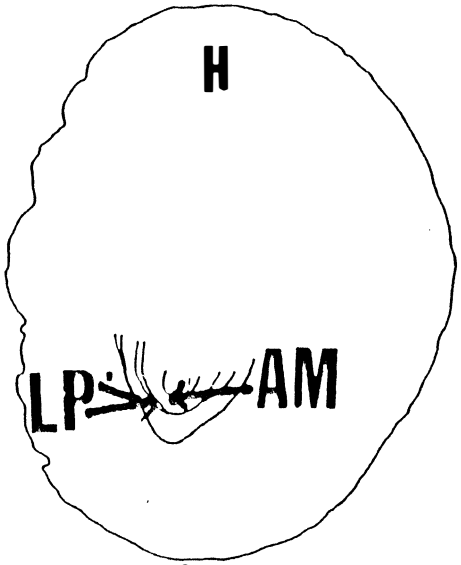
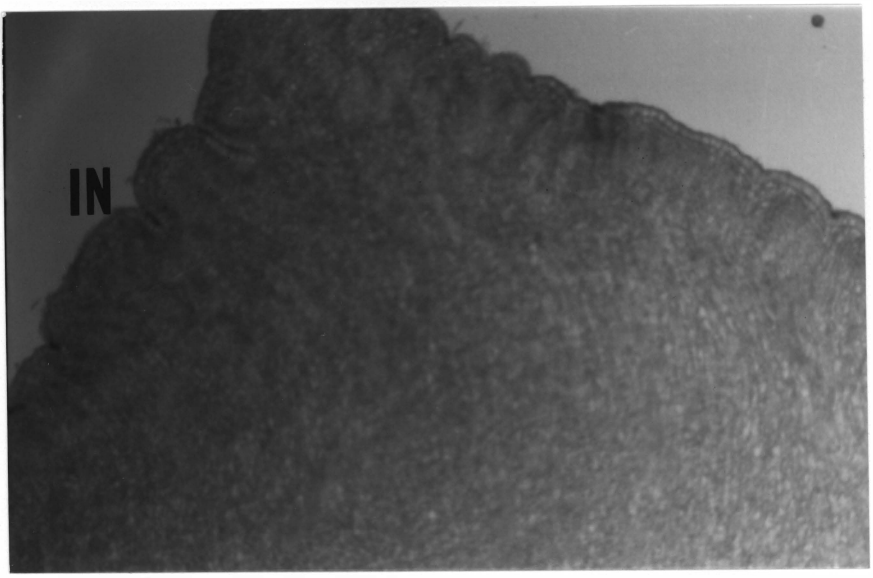

H
LP
AM

Plate 3: Photograph of a longitudinal section of the embryo of Cocos nucifera, showing nodular indentations (IN).

Plate 4: Photograph of a longitudinal section of the embryo of Cocos nucifera, showing procambial strands (PS).


IN

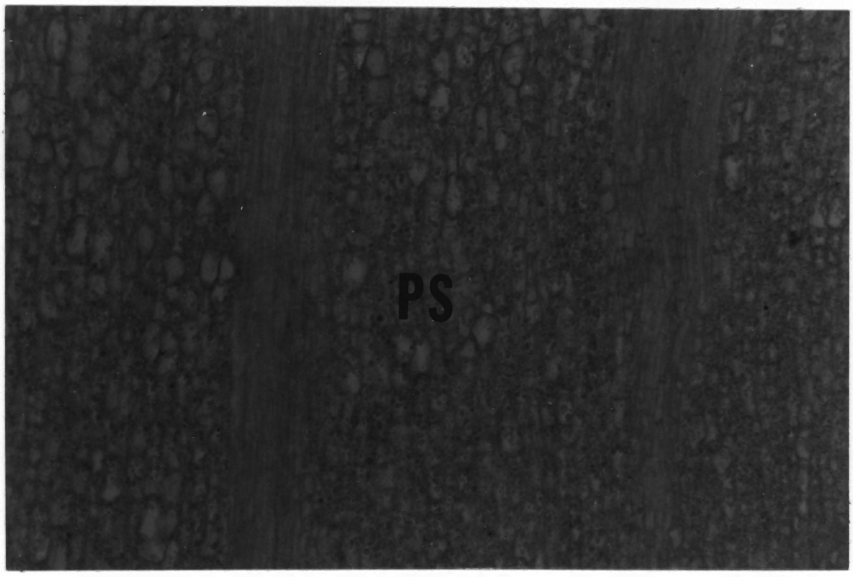
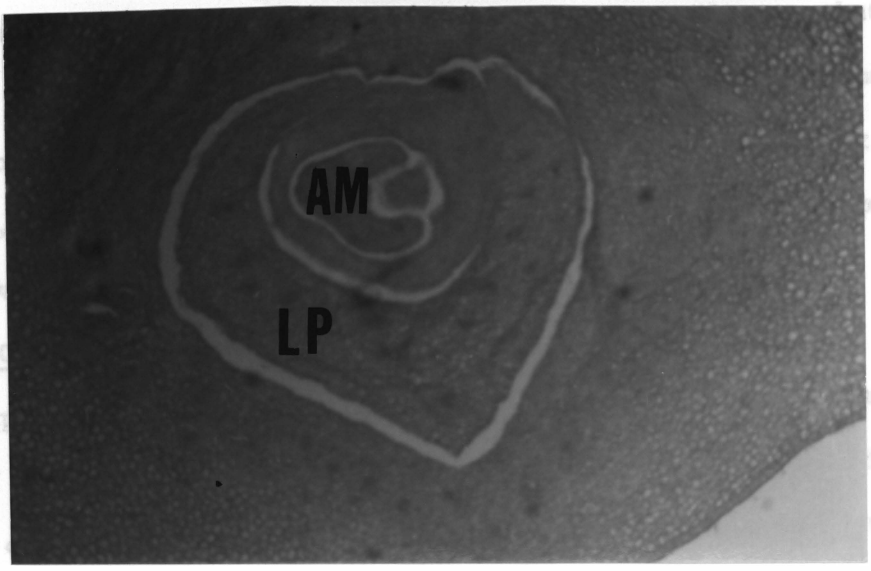
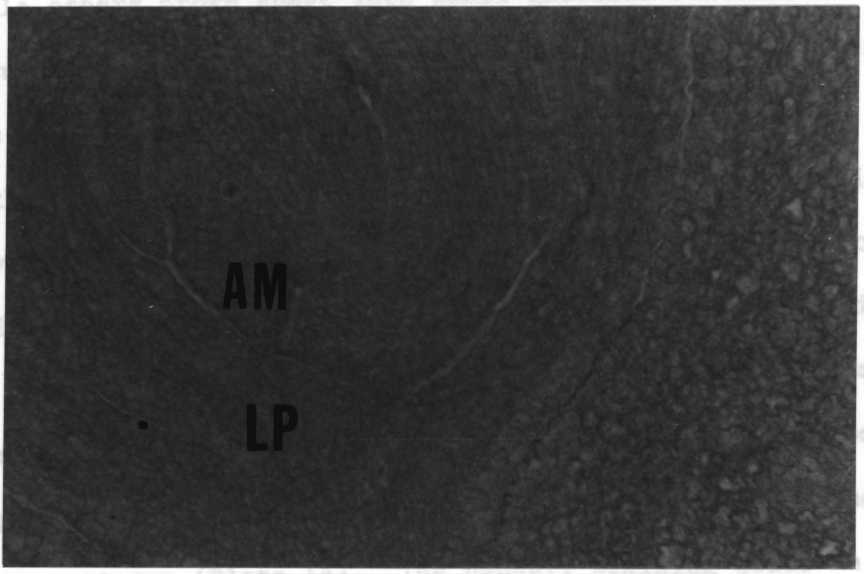

PS

Plate 5: Photograph of a longitudinal section of the embryo of Cocos nucifera showing the apical meristem (AM) and leaf primordia (LP).

Plate 6: Photograph of a cross section of the embryo of Cocos nucifera showing apical meristem (AM) and leaf primordia (LP).


AM
LP


AM
LP

Cells comprising the procambial strands are approximately 5 micrometers in diameter.

With the exception of size and the position of the shoot meristem, it appears that the embryo of Cocos nucifera is similar to that of Phoenix dactylifera. No evidence suggests a separate meristematic region other than the "root pole" for primary root development. However, the meristematic region responsible for shoot development is well developed.

TISSUE CULTURE

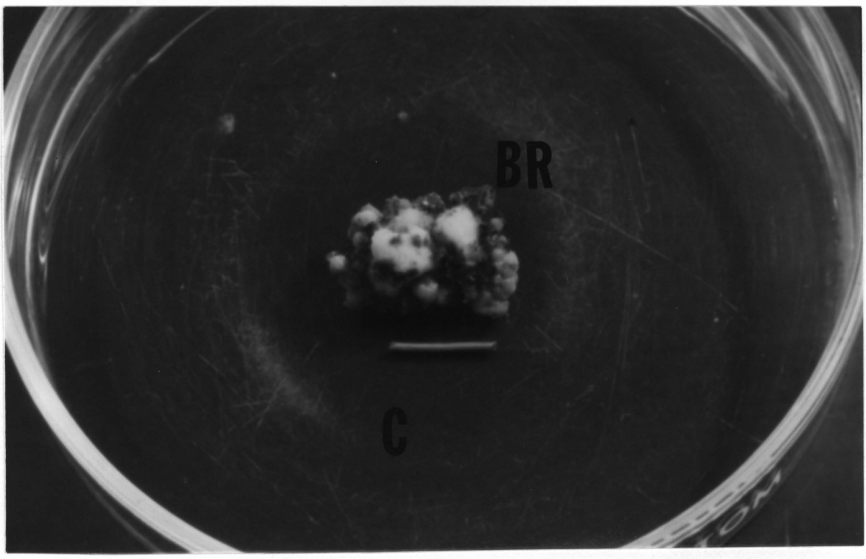
Explanted embryos produced calli within 10-12 weeks in culture. Most of the explants survived, although some were discarded either as a result of contamination or due to their inability to grow on the medium. Surviving embryo explants produced profuse calli and developed a brown color with age (Plate 7). The general growth and appearance of various calli was markedly different. Some calli were compact (Plate 7), while others broke apart into small globular structures at the time of subculture (Plate 8). Some calli produced fingerlike projections (Plates 8 and 9). A cross section of callus tissue revealed a mass of cells that stained densely at the peripheral regions (Plate 10). These densely stained cells are possibly filled with phenolic compounds.

Eight to ten week old calli were subcultured onto stem induction medium where they developed root-like outgrowths (Plates 11 and 12). These root-like outgrowths were anatomically analogous to roots, showing procambial tissue (Plate 13), an apical meristem, and a definite root cap (Plate 14). The densely staining cells in the root cap could possibly be mucilaginous (Tomlinson, 1961; Plate 14).

Two of the calli transferred onto stem induction medium became

Plate 7: Photograph of a compact callus (C) accompanied by browning (BR). (Bar = 1cm)

Plate 8: Photograph of callus in culture showing separated globular structures (GS) and fingerlike projections (FP).


BR
C

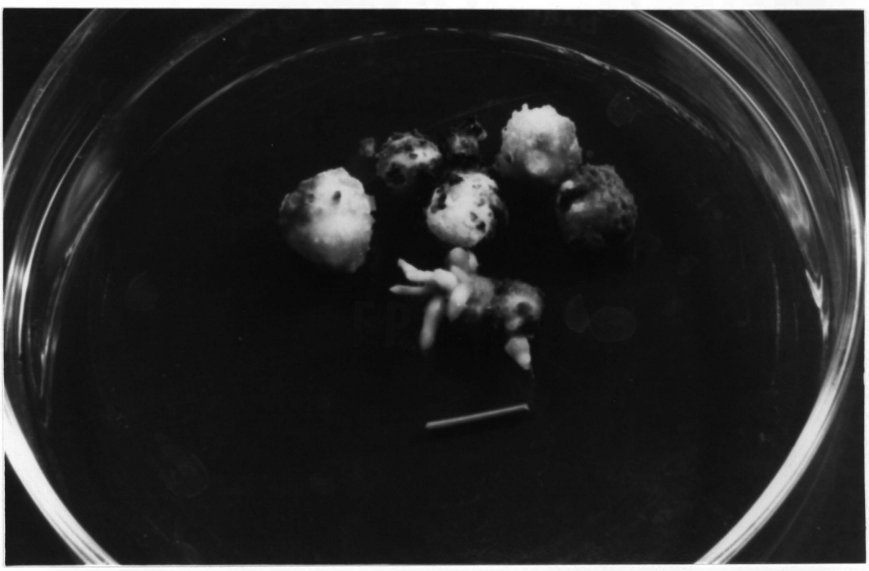
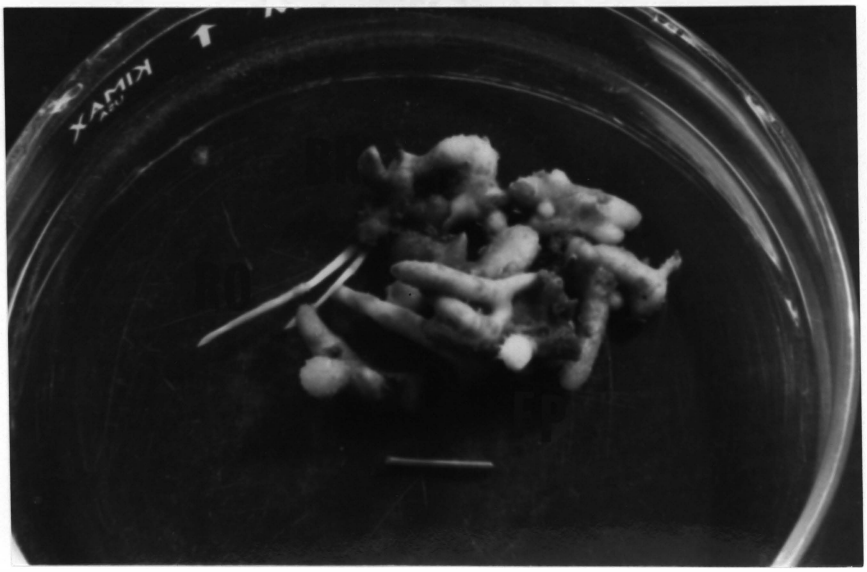


Plate 9: Photograph of callus in culture showing fingerlike projections (FP), rootlike outgrowths (RO), and tissue browning (BR). (Bar = 1cm)

Plate 10: Photograph of a portion of a cross section of callus showing a mass of cells (C) and peripheral stained cells (SC).



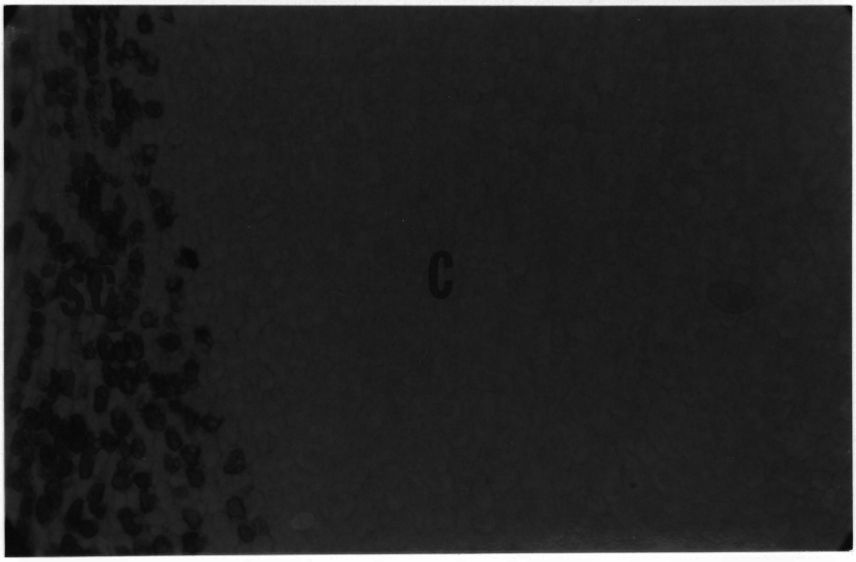

C

Plate 11: Photograph of callus showing differentiation into rootlike outgrowths (RO) (Bar = 1cm)

Plate 12: Photograph of callus in culture producing a root (R). (Bar = 1cm)

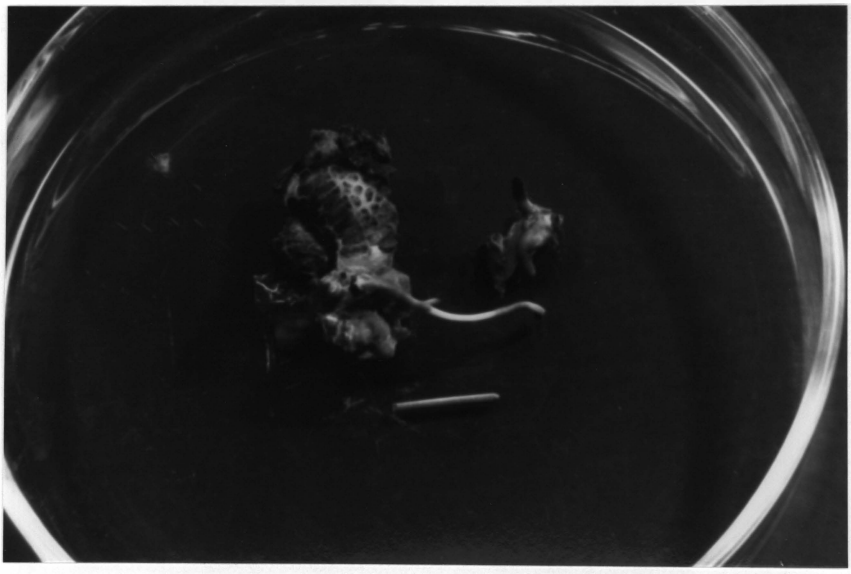
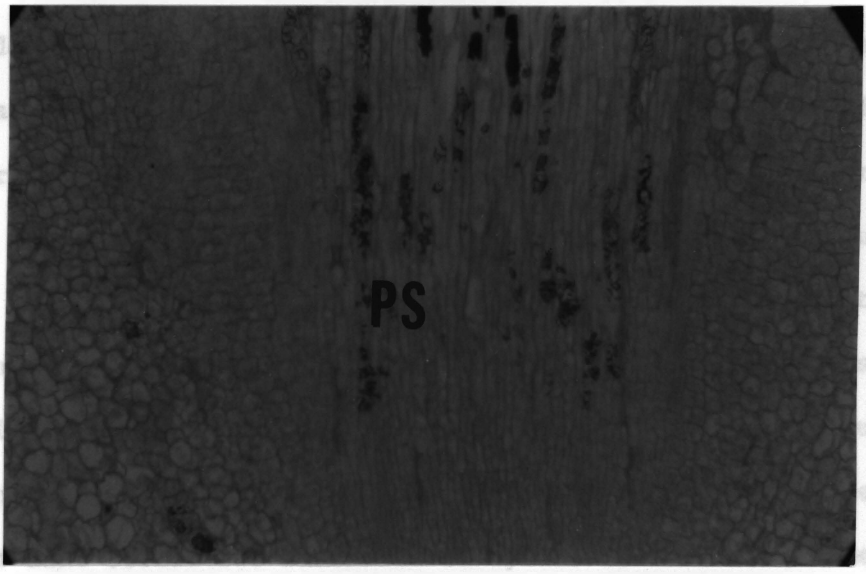
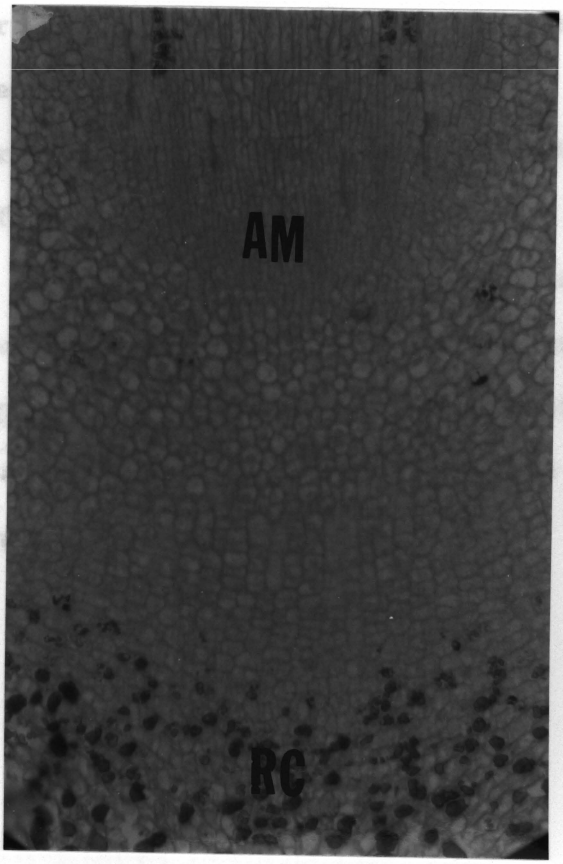


Plate 13: Photograph of a longitudinal section of a root produced in culture showing procambial strands (PS)

Plate 14: Photograph of a longitudinal section of a root produced in culture showing apical meristem (AM) and root cap (RC).


PS


AM
RC

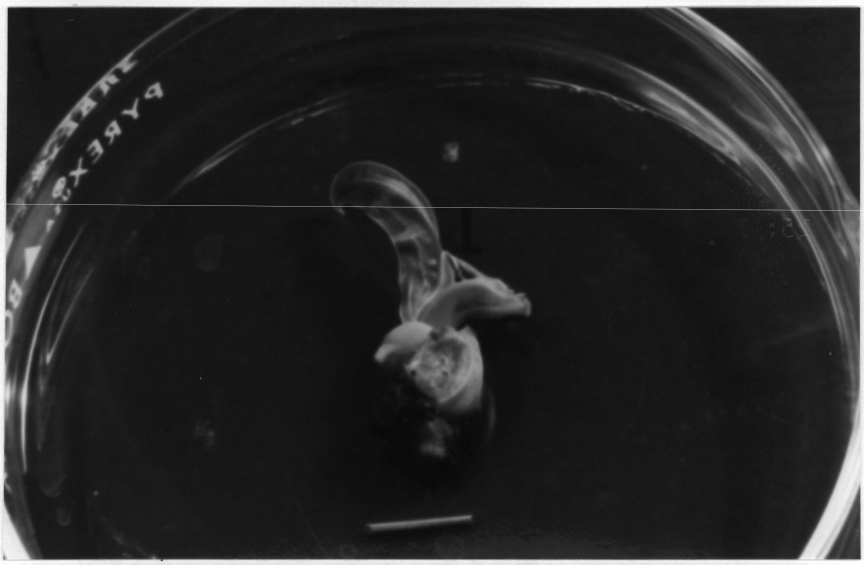
photosynthetic and produced leaves (Plate 15). A cross section of the leaf showed a cutinized epidermis with the underlying hypodermal region being made up of a continuous layer of expansion cells (Tomlinson, 1961; Plate 16). These expansion cells vary considerably in size, being larger between adjacent veins (Plates 16 and 17). The proposed function of these cells is to bring about the expansion of maturing leaves. Tannin-containing cells and vascular bundles are scattered throughout the mesophyll. The general anatomy of the leaf fits the description detailed by Tomlinson (1961) except that both the upper and lower hypodermis was found to be a single layer of cells (Plate 16). Some root like structures resembled embryoids in culture (Plates 18 and 19). However, in longitudinal sections of the organ, cross sections of interconnecting primary root primordia with well-developed tracheary elements were observed (Plates 20 and 21).

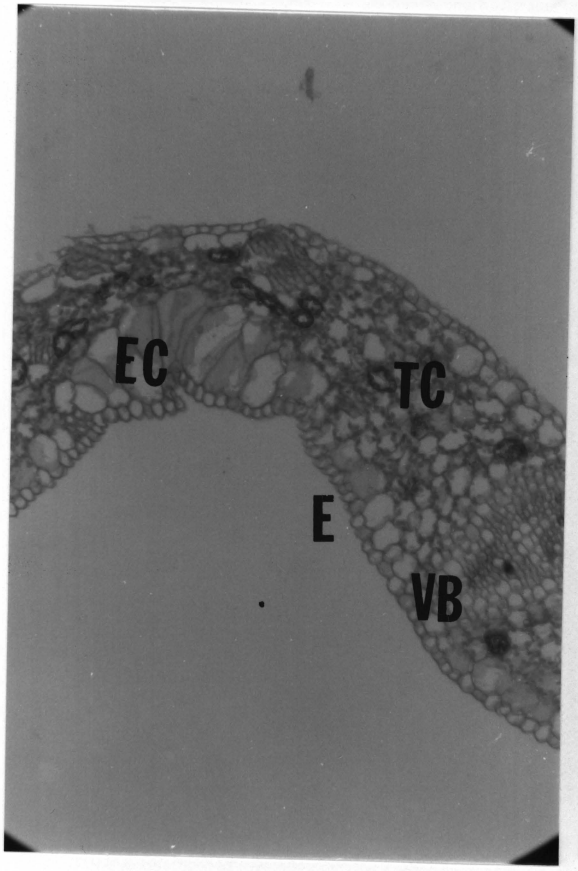
Embryoids were not produced in culture during this study. Success was limited to differentiation of explants and calli into definite roots and leaves. The leaf anatomy is especially characteristic of Cocos, showing a continuous layer of hypodermal expansion cells underlying both upper and lower epidermis of the leaf (Tomlinson, 1961). Improved techniques would eventually lead to the production of embryoids and plantlets in culture.

Plate 15: Photograph of callus in culture differentiated into leaves (L) (Bar = 1cm)

Plate 16: Photograph of a cross section of a leaf produced in culture showing epidermis (E), expansion cells (EC), tannin cells (TC), and vascular bundle (VB).

Plate 17: Photograph of a cross section of a leaf produced in culture, focusing on expansion cells (EC).




EC
TC
E
VB

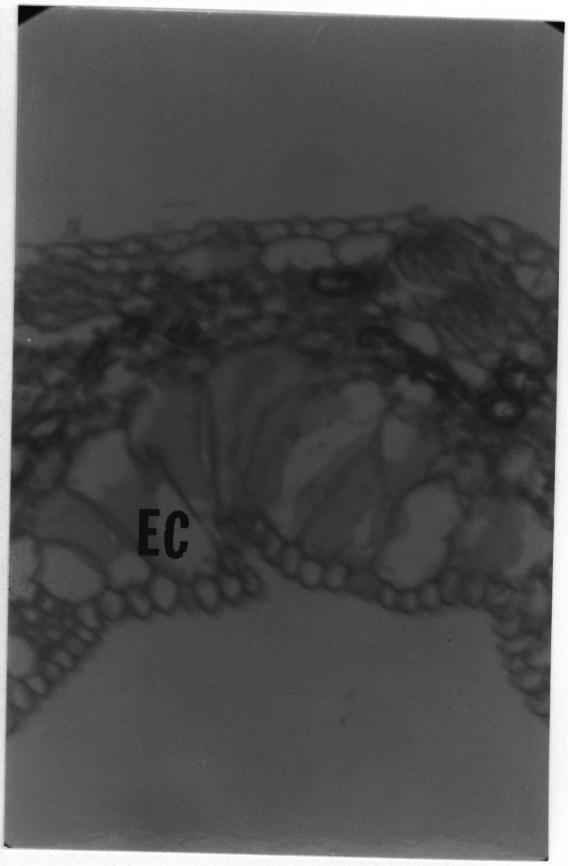

EC

Plate 18: Photograph of embryoid-like structures produced in culture (EM) (Bar = 1cm)

Plate 19: Photograph of an embryoid-like structure produced in culture (Bar = 1cm)

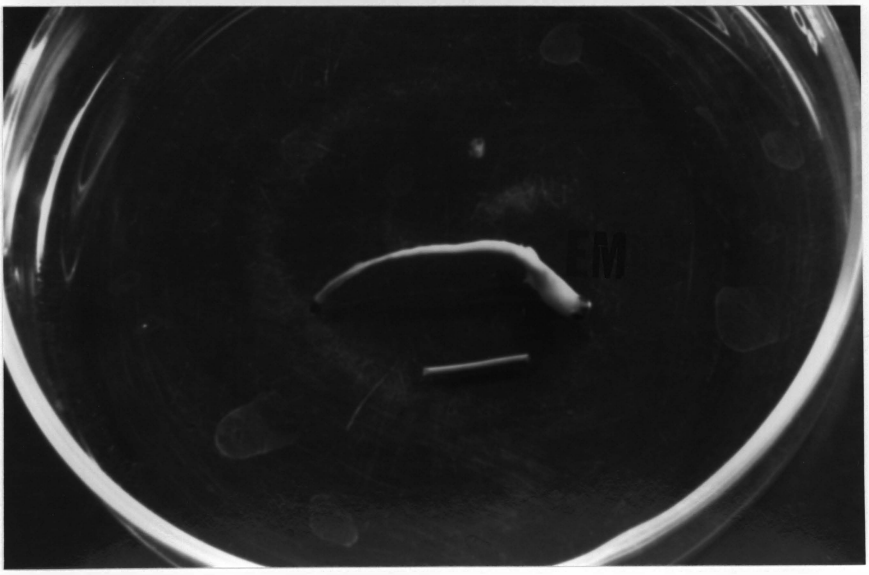
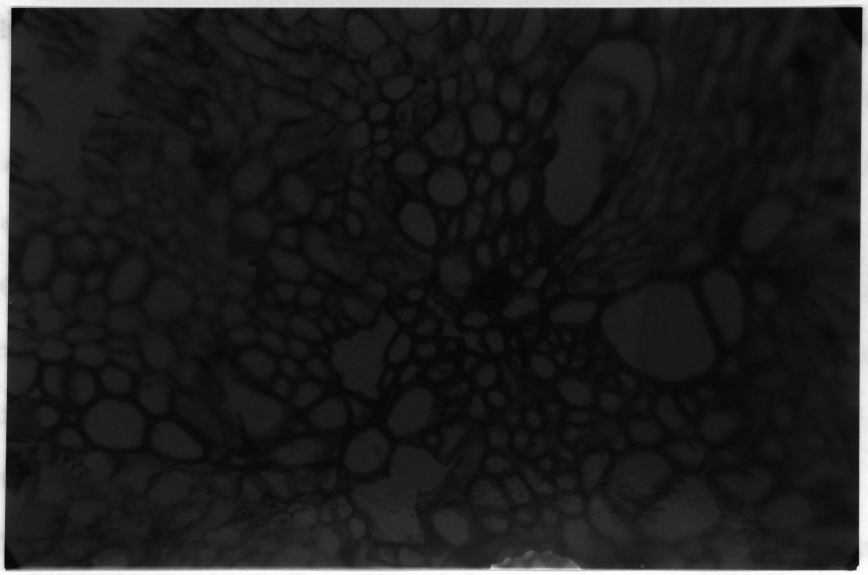
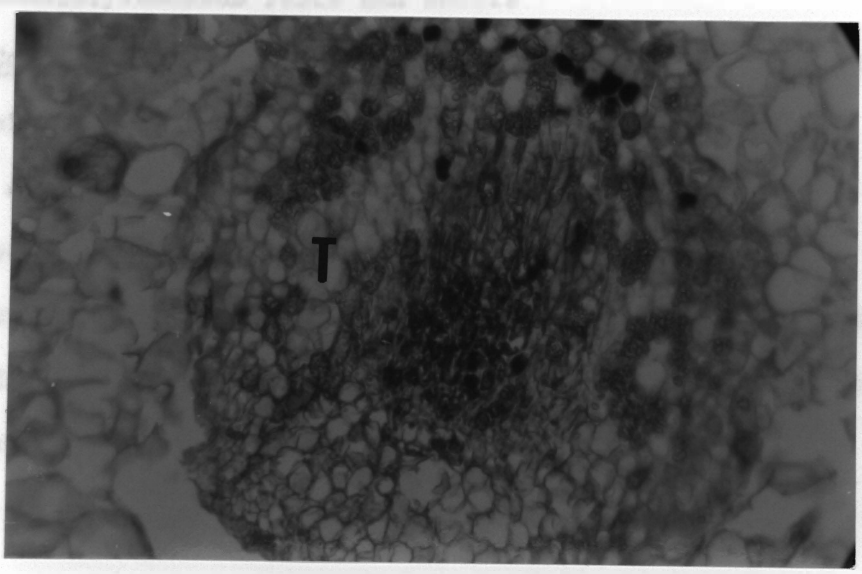


Plate 20: Photograph of a longotudinal section of the embryoid-like structure showing tracheary elements (T) in cross section.

Plate 21: Photograph of a longitudinal section of embryoid-like structure showing origins of tracheary elements of primary root (T)




T

CONCLUSION

The basic MS mineral formulation, coupled with the addition of carbohydrates, vitamins and phytohormones, favors the development of calli on an agar based medium. Callus initiation did not require light, the explants produced successful calli when incubated in the dark at 20°C.

Cross and/or longitudinal sectioning of explant material does not appear to be a requirement for callus formation and differentiation. Juvenile roots and shoots were obtained from both cross and longitudinally sectioned explants.

The light intensity, photoperiod, temperature and relative humidity regulated in the growth chamber was adequate for differentiation of callus into roots and shoots. The roots and shoots obtained in culture were histologically analogous to those of:

1. monocotyledonous roots and shoots
2. Cocos nucifera L., particularly the presence of a continuous layer of hypodermal expansion cells.

Any single callus differentiated into either roots or shoot. Almost all calli developed roots, whereas shoot production was successful in only two calli. Embryoids and free-living plantlets did not develop. It appears that cultural techniques need to be modified in order to obtain embryoids and free-living plantlets.

LITERATURE CITED

Beccari, O. 1917. The origin and disppersal of Cocos nucifera. The Philippine Journal of Science. 12: 27-43.

Branton, R.L., and J. Blake. 1983. Development of organized structures in callus derived from explants of Cocos nucifera L. Annals of Botany. 52: 673-678.

Corner, E.J.H. 1966. The Natural History of Palms. Berkeley and Los Angeles: University of California Press.

Cutter, V.M., and K.S. Wilson. 1954. Effects of coconut endosperm and other growth stimulants upon the development in vitro of embryos of Cocos nucifera. Botanical Gazette. 115: 234-240.

De Guzman, E.V., A.G. Del Rosario, and E.C. Eusebio. 1971. The growth and development of coconut "makapuno" embryo in vitro. III. Resumption of root growth in high sugar media. The Philippine Agriculturist 53(10): 556-579.

Eeuwens, C.J. 1976. Mineral requirements for growth and callus initiation of tissue explants excised from mature coconut palms (Cocos nucifera) and cultured in vitro. Physiologia Plantarum 36: 23-28.

Eeuwens, C.J. 1978. Effects of organic nutrients and hormones on growth and development of tissue explants from coconut (Cocos nucifera) and date palm (Phoenix dactylifera) palms cultured in vitro. Physiologia Plantarum 42: 173-178.

Fisher, J.B., and J.H. Tsai. 1978. In vitro growth of embryos and callus of coconut palm. In Vitro 14: 307-311.

Gupta, P.K., S.V. Kendurkar, V.M. Kulkarni, M.V. Shirgurkar, and A.F. Mascarenhas. 1984. Somatic embryogenesis and plants from zygotic embryos of coconut (Cocos nucifera L.) in vitro. Plant Cell Reports 3: 222-225.

Kyte, L. 1987. Plants From Test Tubes: An Introduction to Micropropagation. Timber Press, Portland, Oregon.

Ochse, et al., 1961. Tropical and Subtropical Argiculture. The Macmillan Company, New York.

Paranjothy, K. 1984. Oil palm. Handbook of Plant Cell Culture: edited Ammirato et al., 3: 591-605.

Purseglove, J.W. 1972. Tropical Crops: Monocotyledons 2. John Wiley and Sons INC, Halsted Press Division, New York.

Reynolds, J.F., and T. Murashige. 1979. Asexual embryogenesis in callus culture of palms. In Vitro 15: 383-387.

Tisserat, B. 1979. Propagation of Date Palm (Phoenix dactylifera L.) in vitro. Journal of Experimental Botany 30: 1275-1283.

Tisserat, B., and D.A. DeMason. 1980. A Histological study of development of adventive embryos in organ cultures of Phoenix dactylifera L. Annals of Botany 46: 465-472.

Tisserat, B. 1984. Date Palm. Handbook of Plant Cell Culture: edited Ammirato et al., 3: 505-545.

Torrey, J.G. 1973. Tissue Culture: Methods and Applications. edited Kruse and Patterson, Academic Press, New York.